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Microplate quantification of plant leaf superoxide dismutases

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Abstract

Superoxide dismutases (SODs) catalyze the dismutation of superoxide radicals in a broad range of organisms, including plants. Quantification of SOD activity in crude plant extracts has been problematic due to the presence of compounds that interfere with the dose–response of the assay. Although strategies exist to partially purify SODs from plant extracts, the requirement for purification limits the rapidity and practical number of assays that can be conducted. In this article, we describe modification of a procedure using *o*-dianisidine as substrate that permits relatively rapid quantification of SOD activity in crude leaf extracts in a microplate format. The method employs the use of a commercial apparatus that permits lysis of 12 tissue samples at once and the use of Pipes buffer to reduce interference from compounds present in crude leaf extracts. The assay provided a linear response from 1 to 50 units of SOD. The utility of the assay was demonstrated using tissue extracts prepared from a group of taxonomically diverse plants. Reaction rates with tissue extracts from two grasses were linear for at least 60 min. Tissues of certain species contained interfering compounds, most of which could be removed by ultrafiltration. The presence of plant catalases, peroxidases, and ascorbate in physiological quantities did not interfere with the assay. This approach provides a means to quantify SOD activity in relatively large numbers of plant samples provided that the possibility for the presence of interfering compounds is considered. The presence of interfering compounds in certain plant tissues necessitates caution in interpreting the effects of plant stresses on SOD. Published by Elsevier Inc.

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Dismutation of superoxide into hydrogen peroxide and oxygen is a primary cellular defense employed by plants to prevent undesirable biological oxidations by oxygen radicals generated during cellular metabolism [1]. Superoxide dismutases (SODs)¹ are the family of enzymes that catalyze this reaction. Direct quantification of SOD activity has been confounded by the instability of the substrate, the superoxide radical. Consequently, quantification of SOD activity often involves an indirect approach based on SOD-mediated inhibition of a redox reaction that involves superoxide [2,3]. The most com-

mon form of this assay is based on competition between the reduction of ferricytochrome c by the superoxide radical and the SOD-catalyzed dismutation of superoxide. Methods based on SOD-mediated inhibition of auto-oxidation reactions also have been described [4–6]. These indirect or "negative" assays occasionally experience false positives due to contamination of reagents with extraneous SOD or substances other than superoxide that contribute to ferricytochrome reduction or auto-oxidation [6,7]. A microplate SOD assay employing a nonenzymatic superoxide generator that found utility in SOD quantification in mammalian tissues also has been described [8]. Its utility in quantifying plant SODs remains unknown.

"Positive" assays that use a photochemical augmentation process in which SOD increases the rate of oxida-

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¹ Abbreviations used: SOD, superoxide dismutase; Pipes, piperazine-*N*, *N'*-bis(2-ethanesulfonic acid); PVPP, polyvinylpyrrolidone.

tion of riboflavin-sensitized o-dianisidine have been developed and modified [9,10]. The latter study adapted the procedure to a microplate format and validated its use by comparison with a negative assay based on auto-oxidation of pyrogallol [5]. The microplate method used in that study required partial purification of SOD with organic extraction, gel filtration, and preparative electrophoresis. The enzyme purification steps, rather than the assay format, provided the limiting factor in high-throughput SOD assay development.

While characterizing antioxidant enzymes from the thermophilic grass, *Dichanthelium lanuginosum* var. *sericeum* (Schmoll), we used the method of Yeh and Kuo [10] to quantify SOD activity in unpurified leaf extracts. The presence of leaf tissue extract in the reaction mixture severely altered the slope of the SOD dose—response curve, suggesting the presence of interfering compounds in leaf tissue from this grass. We modified their method and examined the utility of these modifications for SOD quantification in leaf tissues from taxonomically diverse plants. The modified approach was suitable for many of the tissues tested, but additional fractionation steps were needed to remove interfering compounds from some leaf extracts.

Materials and methods

Growth of plant materials

D. lanuginosum, Lolium perenne L. cv Morning Star, Triticum aestivum L. cv Katepwa, Arabidopsis thaliana L. var. Columbia, Nicotiana benthamiama L., and Lycopersicon esculentum Mill. cvs "Chatham" and "Moneymaker" were grown in 10 × 10 cm plastic pots containing sandy loam with weekly applications of 20:20:20 N:P:K fertilizer. Plants were maintained in a greenhouse with 12h supplemental light (300 μmol/m²/s). Zea mays L. was field-grown at the Oregon State University Botany and Plant Pathology Research Farm.

Protein extraction from plant tissues

Tissues from fully expanded penultimate leaves (40–80mg) were excised, weighed, cut to 0.25–0.5 cm strips, placed into 1.0 ml cooled maceration buffer (components described below) in a lysing matrix E maceration vial (Bio 101, Carlsbad, CA, USA), and maintained on crushed ice. Tissues were macerated using a FastPrep FP120 instrument (Bio 101) at a speed setting of 5.5 for 45 s. The leaf homogenates were centrifuged for 10 min at 12,000g, and supernatants were transferred to microfuge tubes and centrifuged at 14,000g for an additional 30 min. Decanted supernatants were maintained for up to 6 h at 6 °C or were used immediately for total protein and SOD activity quantification. Total protein

was measured according to the manufacturer's protocol 1 of the *NI* Protein Assay Kit (Geno Technology, St. Louis, MO, USA).

Conduct and optimization of SOD assay

Standard reference lines were prepared using bovine liver SOD (Sigma-Aldrich, St. Louis, MO, USA) in which, according to the manufacturer, one unit of SOD corresponded to the amount of enzyme that inhibited the reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 and 25 °C. Triplicate 20 µl aliquots of SOD standards or crude plant protein extracts were placed in consecutive wells of a 96-well microplate, followed by the addition of 125 μl of freshly prepared reaction solution containing K₂PO₄ or piperazine-N, N'-bis(2-ethanesulfonic acid) (Pipes) [10] buffers, pH 7.5, supplemented with 0.4 mM o-dianisidine, 0.5 mM DTPA, and 26 µM riboflavin. The absorbance at $450 \,\mathrm{nm}$ (A₄₅₀) was measured immediately as t=0 min followed by illumination with a fluorescent light placed 12cm above the plate. A₄₅₀ was measured again at $t=30 \,\mathrm{min}$, and regression analysis was used to prepare a standard line relating SOD activity to the change in A₄₅₀. Extrapolated values of SOD were expressed as units SOD per milligram of total protein.

Impact of buffer composition and strength and polyvinylpyrrolidone concentration on SOD assay

The effects of buffer composition and concentration on SOD activity were quantified by conducting leaf protein extractions in K_2PO_4 and Pipes buffers ranging in concentrations from 10 to 100 mM. SOD was quantified in leaf protein extracts spiked with 0–50 units/ml of bovine liver SOD, extrapolated values of SOD were calculated as units SOD per milligram total protein, and regression lines relating SOD activity to the change in A_{450} were prepared. These regression lines were compared with that of the standard reference line that quantified authentic SOD suspended in buffer in the absence of plant extracts. The effects of maceration buffer supplemented with specified quantities of polyvinylpyrrolidone (PVPP) concentrations were measured using a similar protocol.

Statistical evaluation of regression lines

Regression analyses were conducted using Statgraphics Plus for Windows version 4.0 (Manugistics, Rockville, MD, USA). A standard reference line using triplicate wells with 1–50 units/ml of bovine liver SOD was included on each microplate on which leaf SOD was quantified. The equation of the reference line was compared with the regression lines derived from experimental leaf extracts spiked with identical quantities of

bovine liver SOD. Differences in the *y* intercept between the standard line and those developed with leaf extracts were used as a measure of endogenous plant SOD. Minimization of deviations of experimental slopes from that of the reference line were used as criteria for optimizing SOD assay conditions.

Impact of ascorbate, peroxidase, and catalase on SOD quantification

Experiments were conducted to determine whether the presence of ascorbate, peroxidase, and/or catalase in physiological concentrations affected SOD quantification. The effect of ascorbate was determined comparing a standard SOD reference line derived using 0-50 units of SOD activity with a line derived from 0 to 50 units of SOD activity amended with 10 or 100 μM sodium L-ascorbate (Sigma–Aldrich). The impacts of peroxidase and catalase were evaluated by comparing SOD reactions using standard substrate and substrate solutions preincubated with either 0-50 units of peroxidase (Sigma-Aldrich) or 0-44.6 units of catalase (Sigma-Aldrich). Catalase activity in D. lanuginosum and L. esculentum leaves was quantified using a colorimetric assay according to the manufacturer's instructions (Sigma-Aldrich). Leaf extracts from L. esculentum were partitioned into 30K molecular weight cutoff fractions using ultrafiltration methods described below.

Evaluation of assay to quantify SOD activity in extracts from taxonomically diverse plants

To evaluate the utility of this approach to quantify SOD in a broader range of plants, the impact of leaf extracts prepared from monocotyledonous and dicotyledonous plants on standard SOD reference lines was determined. Plots of observed versus predicted SOD values were prepared to determine the suitability of the assay to quantify SOD in these plants.

Ultrafiltration to remove interfering compounds

Leaf protein extracts (250 µl) were placed into a Micron centrifugal filter device (Millipore, Bedford, MA, USA) with selected molecular weight cutoff limits and were centrifuged for 10 min at 12,000 g. An additional 250 µl of 50 mM Pipes (pH 7.5) was added, and the filter was centrifuged for an additional 10 min at 12,000 g. The retentate was resuspended to the original volume (250 µl) with Pipes buffer, and SOD was quantified and compared with that of unfiltered extract.

SOD activity gels

To provide a visual check on the relative accuracy of the microplate assay, SOD was quantified in leaf protein extracts that were subsequently electrophoresed in precast 4–16% native acrylamide gels (BioWhittaker, Rockland, ME, USA), and SOD activity was visualized [11].

Results

Impact of plant extract on SOD dose-response

Regression analysis of SOD dose-response data showed that a sigmoidal dose-response of the form y = a + b(square root (x)) ($r^2 = 0.97$) provided the best fit within the range of 1-100 units of authentic bovine liver SOD (data not shown), whereas a linear model of the form y = a + bx ($r^2 = 0.96$) was most appropriate within the range of 1-50 units. We chose the linear model for subsequent analyses of leaf extracts because SOD activity measurements usually fell within the range of 1– 50 units/mg leaf extract protein. When D. lanuginosum leaf extract in 10mM phosphate buffer (pH 7.5) was added to the bovine SOD solutions, the slope of the regression line was altered significantly (P < 0.0001) from that of the standard reference line, suggesting that plantderived compounds interfered with the expected doseresponse (Table 1).

Impact of buffer on interference associated with plant extract

To determine whether changes in buffer concentration reduced this interference, dose–response trials using concentrations of phosphate buffer ranging from 10 to 100 mM were conducted. For comparison, the assay also was conducted using identical concentrations of Pipes, a zwitterionic buffer. Altering phosphate concentrations between 10 and 100 mM had no significant impact on the slope of regression lines (data not shown) or on the response of the standard curve to the presence of leaf

Table 1 Impact of leaf extract on slopes of SOD dose–response regression lines

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Treatment	Slope ^a	Pct. change in slope ^b
KPO ₄ buffer (10 mM, pH 7.5)	1.766 ± 0.067	
Leaf extract in KPO ₄ buffer	3.068 ± 0.189	73***
Pipes buffer (10mM, pH 7.5)	2.697 ± 0.086	
Leaf extract in Pipes buffer	2.834 ± 0.099	5 ^{ns}

Note. An assay using a photochemical augmentation process, where SOD increased the rate of oxidation of o-dianisidine, was used to quantify authentic bovine liver SOD in phosphate or Pipes buffers in the absence and presence of 2.5 mg/ml of leaf extract from *D. lanuginosum*, a thermophilic grass. Assay mixtures (pH 7.5) contained 0–50 units of SOD, 0.4 mM o-diansidine, 0.5 mM DTPA, and 26 μ M riboflavin.

^a The slope was calculated by regression analysis using the equation y = mx + b, where $y = \Delta A_{450}$ at $30 \min \times 1000$, m = slope of regression line, x = SOD units, and b = y intercept.

^b Percentage change in slope associated with presence of plant extract (***Significant at P < 0.0001; ^{ns}Not significant).

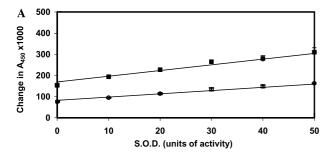
extract. In contrast, when Pipes was used in place of phosphate, *D. lanuginosum* leaf extract had no significant effect on the slope of the dose–response (Table 1 and Fig. 1). Plots of predicted versus observed SOD units showed that 50 mM Pipes (pH 7.5) consistently provided a slope closest to 1 (data not shown), and this buffer was used in subsequent studies. Reaction rates with *D. lanuginosum* and *L. perenne* extracts were linear for at least 60 min (data not shown).

Impact of PVPP on interference associated with plant extract

The addition of PVPP to the leaf extraction buffer significantly reduced interference associated with the presence of *D. lanuginosum* leaf extract (Table 2). All subsequent SOD assays were conducted using 100 mg/ml PVPP in the leaf extraction buffer.

Comparison of electrophoretic and microplate estimates of plant SOD activity

The microplate assay was used to quantify SOD activity in *D. lanuginosum* leaves harvested from plants propagated at 25 and 45 °C, and it failed to detect SOD activity in leaves from plants grown at the higher temperature. When these same leaf extracts were electrophoresed and stained for SOD activity, significant SOD



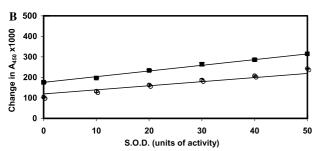


Fig. 1. Regression lines to compare phosphate and Pipes buffers for utility in quantification of SOD in the presence of leaf-derived interfering compounds. Specified quantities of bovine liver SOD were suspended in 50 mM buffer (pH 7.5) and were quantified in the presence and absence of leaf extracts from *D. lanuginosum*. (A) KPO₄ buffer. \bigcirc , no leaf extract; \blacksquare , leaf extract containing 2.5 mg/ml leaf protein present in assay. (B) Pipes buffer. \bigcirc , no leaf extract; \blacksquare , leaf extract containing 2.5 mg/ml leaf protein present in assay. Data points represent means \pm standard deviations (n = 3).

Table 2 Impact of PVPP on plant leaf SOD dose–response regression line

Treatment	Slope ^a	Pct. change in slope ^b
Pipes buffer	2.829 ± 0.201	_
Leaf extract with 0 mg/ml PVPP	4.164 ± 0.285	47***
Leaf extract with 50 mg/ml PVPP	3.660 ± 0.149	29 ^{ns.}
Leaf extract with 100 mg/ml PVPP	2.958 ± 0.162	5 ^{ns.}

Note. Standard curves quantifying 0–50 units of authentic bovine liver SOD in 50 mM Pipes buffer (pH 7.5) were compared with curves developed after authentic SOD standards were spiked with leaf extracts from *D. lanuginosum*. Slopes of regression lines were compared by two-way analysis of variance. Regression analyses of SOD activity as a function of the change in A₄₅₀ were conducted where data points represented the mean of three replicate determinations.

- ^a Slopes of regression lines were calculated using the equation y = mx + b, where $y = \Delta A_{450}$ at $30 \min \times 1000$, m = slope, x = SOD units, and b = y intercept.
- ^b Percentage change in slope associated with presence of plant extract (***Significant at P < 0.0001; ^{ns}Not significant).

activity was detected (Fig. 2). Ultrafiltration of leaf extracts from *D. lanuginosum* plants propagated at 35°C partially restored apparent SOD activity (Table 3). The addition of leaf extract ultrafiltrate (30kDa cutoff filter) from temperature-treated *D. lanuginosum* reduced the apparent enzyme activity of authentic bovine SOD measurable by the microplate assay (data not shown).

Utility of assay to quantify SOD from taxonomically diverse plants

Bovine liver SOD was quantified in the presence of leaf extracts from taxonomically diverse plants to deter-

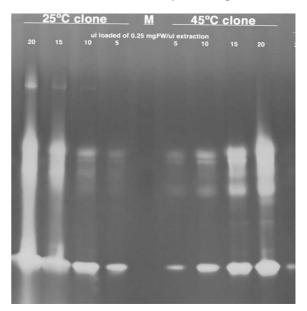


Fig. 2. Electrophoretic analysis of the impact of temperature on SOD activity in leaf tissue from *D. lanuginosum*. Tissue from new leaves that developed during propagation at 25 or 45 °C was macerated in phosphate buffer, electrophoresed in native 4–15% acrylamide gradient gels, and stained for SOD activity using the method of Beauchamp and Fridovich [12].

Table 3 Molecular weight cutoff ultrafiltration to remove *D. lanuginosum* leaf compounds produced at elevated temperatures that interfere with SOD quantification

Molecular weight cutoff filtration	Plant growth temperature (°C)	Predicted SOD activity (units/mg total protein) ^a	95% LSD test homogeneous groups ^b
None	Greenhouse	62.9	С
	35/25	-212.3	A
30,000	Greenhouse	81.2	C
	35/25	12.2	В

Note. Leaf extract prepared in 50 mM Pipes (pH 7.5) was centrifuged through 30,000 molecular weight cutoff Micron centrifugal filter device, and SOD remaining in the retentate was quantified.

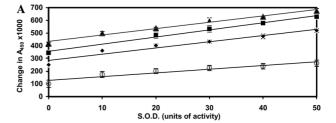
^a Mean values (n = 3) extrapolated from standard curve prepared using 1–50 units of authentic bovine liver SOD. SOD was quantified using a photochemical augmentation process where SOD increased the rate of oxidation of o-dianisidine. Negative extrapolated values indicate the presence of compounds in leaf extracts that reduced the apparent rate of oxidation in the presence of SOD.

^b Tested using Fisher's least significant difference procedure at the 95% confidence level. Values followed by the same letter are not significantly different.

mine whether species-related interfering compounds affected the suitability of the SOD assay. Dose-response curves developed by spiking authentic SOD with leaf extracts from monocotyledonous plants, including wheat (T. aestivum L.), maize (Z. mays L.), and perennial ryegrass (L. perenne L.), had slopes that were not significantly different from standard reference curves that contained no plant extract (Fig. 3A). The slopes of similar curves developed with extracts from certain dicotyledonous plants were not significantly different from the standard reference curve, but the presence of tomato leaf extract in solutions of authentic SOD significantly reduced the apparent SOD activity (Fig. 3B). The diminished SOD estimates in the presence of tomato leaf extract suggested the presence of interfering compounds in tomato. When selected leaf compounds were removed from leaf extracts by ultrafiltration, significant restoration of SOD activity was measured (Table 4). The degree of reduction was dependent on molecular weight cutoff. Use of filters with molecular weight cutoff in excess of 50 kDa caused an apparent loss of SOD through filtration.

Impact of ascorbate, peroxidase, and catalase on SOD quantification

Regression plots of SOD reactions that contained 10 or $100 \,\mu\text{M}$ sodium L-ascorbate were not significantly different (P = 0.10) from standard SOD reference lines (data not shown). Similarly, the addition of 0–50 units of peroxidase activity to the SOD reaction substrate had no significant impact (P = 0.05) on assay accuracy (data not shown). In contrast, the addition of catalase to con-



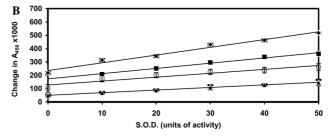


Fig. 3. Quantification of SOD in leaf extracts of taxonomically diverse plants. Leaf extracts (2.5 mg/ml protein) were spiked with known quantities of bovine liver SOD and suspended in 50 mM Pipes buffer (pH 7.5) containing 0.4 mM o-dianisdine, 0.5 mM DTPA, 26 μ M riboflavin, and 100 mg/ml PVPP. (A) Monocotyledonous plants. \bigcirc , SOD standards; \spadesuit , maize (Z. mays); \blacktriangle , perennial ryegrass (L. perenne); \blacksquare , wheat (T. aestivum). (B) Dicotyledonous plants. \bigcirc , SOD standards; \spadesuit , A. thaliania; \blacksquare , N. benthianiama; \blacktriangle , tomato (L. esculentum). Data represent means \pm standard deviations (n = 3).

Table 4 Molecular weight cutoff ultrafiltration to remove L. esculentum leaf compounds that interfere with SOD quantification

Molecular weight cutoff	Predicted SOD activity (units/mg total protein) ^a	95% LSD test homogeneous groups ^b
None	-20.1	A
10,000	-4.1	В
30,000	16.1	C
50,000	28	D
100,000	10.7	C

Note. Leaf extract prepared in 50 mM Pipes (pH 7.5) was centrifuged through specified Micron centrifugal filter devices, and SOD activity in the retentate was quantified.

^a Mean values (n = 3) extrapolated from standard line prepared using 1–50 units of authentic bovine liver SOD. SOD was quantified using a photochemical augmentation process where SOD increased the rate of oxidation of o-dianisidine. Negative extrapolated values indicate the presence of compounds in leaf extracts that reduced the apparent rate of oxidation in the presence of SOD.

^b Tested using Fisher's least significant difference procedure at the 95% confidence level. Values followed by the same letter are not significantly different.

centrations of at least 2.2 units/ml resulted in overestimations of SOD values (Table 5). Electrophoretic analysis of the catalase preparation was conducted to determine whether SOD contamination of the commercial catalase caused the apparent overestimation. No demonstrable SOD (limit of detection = 1 unit) was present, even when 400 units of catalase were loaded on the gel (data not shown). To determine whether endogenous catalase activity in leaves from heat-treated

Table 5
Effect of catalase on SOD quantification

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Catalase (units/ml)	Measured SOD activity (units/ml) ^a	95% LSD test homogeneous groups ^b
0	49	A
0.1	45.9	A
0.2	51.4	A
0.4	52.4	A
0.5	52.6	A
0.7	51.0	A
1.4	53.9	AB
2.2	59	В
4.5	63.4	В
8.9	69.4	C
17.8	96.8	D
31.2	101	DE
44.6	103	E

Note. Catalase prepared in enzyme dilution buffer provided in a commercially available catalase quantification kit was added to SOD reaction substrate containing $50\,\mathrm{units/ml}$ of bovine liver SOD, and change in A_{450} was quantified after $30\,\mathrm{min}$ incubation.

^a Mean values (n = 3) extrapolated from standard reference line prepared in reaction mixture that contained no catalase.

Table 6
Effect of growth temperature on leaf catalase activity in *D. lanugino-sum*, a thermophilic grass

Growth temperature ^a °C	Catalase (activity/mg protein/ml) ^b	
35/25	0.89 ± 0.14	
25/20	0.48 ± 0.11	

^a Plants were acclimated to growth temperatures for at least 21 days prior to collection of leaf tissue for catalase measurements. Temperatures indicate day/night temperatures with 12h daylengths provided at a light intensity of $300 \, \mu mol/m^2/s$.

D. lanuginosum plants was sufficient to partially explain the altered SOD determinations observed, catalase activity was quantified in D. lanuginosum leaf extracts. Catalase activity was greater in D. lanuginosum plants propagated at the higher temperature, but all extracts contained less than 2.2 units of activity/mg protein/ml (Table 6). Although tomato leaf extracts contained compounds that reduced, rather than increased, apparent SOD activity, we also quantified catalase activity in L. esculentum leaves. Catalase concentrations did not exceed 0.38 units of activity/mg protein/ml in either crude extracts or 30 K MWCO retentates or filtrates (Table 7).

Discussion

Increased SOD activity has been correlated with enhanced stress tolerance in a variety of plants [14–16],

Table 7
Quantification of catalase and SOD activities in leaves from *L. esculentum* cv "Moneymaker"

Molecular weight filtration ^a	Catalase (activity/mg protein/ml) ^b	SOD ^c (activity/mg protein/ml)
None #1	0.38	15.72
None #2	0.30	5.34
30kDa retentate #1	0.21	15.80
30kDa retentate #2	0.20	9.27
30kDa filtrate #1	0.03	-48.37
30 kDa filtrate #2	0.05	-46.15

^a Tissue extracts were prepared from fully expanded penultimate leaves. Molecular weight filtration was conducted in 30kDa centrifugal filter devices. After centrifugation, retentates and filtrates were resuspended in original volumes.

^b Catalase activity was quantified using a commercially available colorimetric assay. Values represent means (n = 3).

^c SOD was quantified using a photochemical augmentation process where SOD increased the rate of oxidation of o-dianisidine. Data represent mean values (n = 3) extrapolated from a standard reference plot of A_{450} as a function of SOD activity. Negative extrapolated values indicate the presence of compounds in leaf extracts that reduced the apparent rate of oxidation in the presence of SOD.

and these results have led to the development of genetic technologies to improve crop stress tolerance [17]. A critical factor in evaluating the impact of SOD on stress tolerance is a means to quantify the enzymatic activity in plant tissues. The potentially large number of plant samples required for genetic analyses precludes the use of an approach that requires cumbersome enzyme purification procedures. We found that modification of a previously published approach [8,9], including extraction of leaf SOD in the presence of PVPP and the use of Pipes as an assay buffer, permitted quantification of SOD in crude leaf extracts from selected plants in a microplate format compatible with relatively high-throughput. Our statistical models relating SOD activity to A₄₅₀ are consistent with a previously published microplate approach [9], indicating that our modifications did not adversely affect the utility of the assay.

Buffer effects on baseline absorbance and the portion of photoreduction of Nitro blue tetrazolium (NBT) inhibitable by SOD were noted when SOD was quantified by the NBT reduction method [18], although no data were presented on the impact of buffer on interfering compounds. Some zwitterionic buffers bind copper(II) and have surfactant characteristics that reduce their utility for quantification of copper-containing enzymes, including copper/zinc SOD. A previous study that characterized copper complexation and surfactant properties of three zwitterionic buffers showed no copper complexation by Pipes and low surfactant activity [13]. We observed that Pipes, relative to phosphate, was more suitable for quantification of SODs in extracts that contained plant-derived interfering compounds. Despite modification of the assay by use of PVPP and Pipes, two lines of evidence showed that certain leaf extracts

^b Tested using Fisher's least significant difference procedure at the 95% confidence level. Values followed by the same letter are not significantly different.

^b Catalase activity was quantified in pulverized leaf tissue using a commercially available colorimetric assay. Values given are means of triplicate measurements ± standard errors.

still contained compounds that interfered with SOD activity. First, electrophoretic analysis of extracts from temperature-treated D. lanuginosum leaves demonstrated the presence of SOD activity, even though microplate analysis of the extracts indicated levels of SOD below the detection limit of the assay. One possibility is that inhibiting compounds were sufficiently charged to permit electrophoretic separation from SOD and subsequent restoration of apparent SOD activity in the gel. Second, the addition of tomato leaf extracts to solutions of authentic bovine SOD decreased measurable SOD activity. Ultrafiltration of leaf extracts at least partially restored measurable SOD levels in tomato leaves and leaves from heat-treated D. lanuginosum plants. These assay interferences were in contrast to the lack of interference noted in crude extracts of Escherichia coli and the red alga Porphyridium cruentum used in the original description of the method [8].

The identity and biological significance of compounds that interfere with the SOD-mediated increase in in vitro photoreduction of o-dianisidine remain unknown. It is possible that compartmentalized interfering compounds are released, or are created during the enzyme extraction process, and consequently represent artifacts of the assay procedure. Alternatively, interfering compounds, some of which have apparent molecular weights in excess of 10kDa, may be formed endogenously, in some cases, in response to plant stress. The origin and biological role of these compounds may be of considerable interest in efforts to enhance plant stress tolerance. Although sufficient levels of catalase could increase the rate of oxidation of o-dianisidine, endogenous quantities of catalase appeared to be insufficient to explain the interference noted in heat-treated D. lanuginosum plants.

The utility of this modified microplate method for SOD quantification in relatively large numbers of crude plant leaf extracts has been demonstrated, including its use for some commonly used plant experimental models. It remains critical, however, that the potential for the presence of SOD interfering compounds in leaf extracts be considered when quantifying SOD in unpurified tissues. In some cases, additional purification steps will be required.

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